

RNA-Dependent Replication and Transcription of Hepatitis Delta Virus RNA Involve Distinct Cellular RNA Polymerases

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Cellular DNA-dependent RNA polymerase II (pol II) has been postulated to carry out RNA-dependent RNA replication and transcription of hepatitis delta virus (HDV) RNA, generating a full-length (1.7-kb) RNA genome and a subgenomic-length (0.8-kb) mRNA. However, the supporting evidence for this hypothesis was ambiguous because the previous experiments relied on DNA-templated transcription to initiate HDV RNA synthesis. Furthermore, there is no evidence that the same cellular enzyme is involved in the synthesis of both RNA species. In this study, we used a novel HDV RNA-based transfection approach, devoid of any artificial HDV cDNA intermediates, to determine the enzymatic and metabolic requirements for the synthesis of these two RNA species. We showed that HDV subgenomic mRNA transcription was inhibited by a low concentration of α -amanitin ($<3 \mu\text{g/ml}$) and could be partially restored by an α -amanitin-resistant mutant pol II; however, surprisingly, the synthesis of the full-length (1.7-kb) antigenomic RNA was not affected by α -amanitin to a concentration higher than $25 \mu\text{g/ml}$. By several other criteria, such as the differing requirement for the de novo-synthesized hepatitis delta antigen and temperature dependence, we further showed that the metabolic requirements of subgenomic HDV mRNA synthesis are different from those for the synthesis of genomic-length HDV RNA and cellular pol II transcripts. The synthesis of the two HDV RNA species could also be uncoupled under several different conditions. These findings provide strong evidence that pol II, or proteins derived from pol II transcripts, is involved in mRNA transcription from the HDV RNA template. In contrast, the synthesis of the 1.7-kb HDV antigenomic RNA appears not to be dependent on pol II. These results reveal that there are distinct molecular mechanisms for the synthesis of these two RNA species.

Hepatitis delta virus (HDV) is a subviral particle containing a circular RNA genome of 1.7 kb which resembles plant viroid RNAs and contains ribozyme activities (27). HDV RNA can replicate itself in cultured cells, requiring only a virus-encoded protein, the hepatitis delta antigen (HDAg) (13, 26). HDAg, however, does not possess an RNA polymerase activity. Thus, it has always been assumed that HDV utilizes host cell RNA polymerases to replicate its RNA genome, in a mechanism similar to that of viroid RNA replication (40). However, the dependence of HDV RNA replication on a viral protein (HDAg) distinguishes HDV RNA synthesis from the synthesis of plant viroid RNA, which does not encode any protein. Furthermore, unlike plant cells (41), animal cells are not known to have RNA-dependent RNA polymerases. These issues raised interesting questions concerning which cellular polymerases are responsible for HDV RNA-dependent RNA synthesis and how they are converted from DNA- to RNA-templated polymerases.

The common belief that HDV RNA synthesis is carried out by cellular RNA polymerase II (pol II) came from early in vitro transcription studies using nuclear extracts of HDV-replicating cells, which revealed that HDV RNA synthesis could be inhibited by α -amanitin at concentrations as low as $1 \mu\text{g/ml}$ (31). However, these studies used nuclear extracts from a cell line (H189) (31) that contains an integrated cDNA trimer of HDV

under the control of a foreign promoter. This cDNA first transcribes an HDV RNA, which is then replicated by RNA-dependent RNA synthesis. The presence of HDV cDNA in the assay system rendered it uncertain whether the observed effect of α -amanitin was due to inhibition of pol II-mediated transcription from the HDV cDNA or inhibition of subsequent RNA-dependent RNA synthesis. In fact, when the same experiments were performed using an exogenously added HDV RNA template in an uninfected cell extract, the HDV RNA synthesis was not inhibited by α -amanitin (31). Another piece of evidence in support of the role of pol II in HDV RNA synthesis came from the study using purified pol II and basal transcription factors in a reconstituted in vitro transcription assay, which showed that RNA synthesis from an HDV RNA template was sensitive to α -amanitin (14). This appears to provide the most direct evidence that pol II carries out RNA-dependent synthesis of the HDV RNA species. Unfortunately, this result has so far not been reproduced. Also, in all of these in vitro transcription systems, HDAg was not required and its addition had no effect on HDV RNA synthesis. The absence of a role for HDAg in these assays cast further doubt on the biological relevance of these in vitro studies.

During HDV replication, three HDV RNA species are produced: the 1.7-kb antigenome, the 1.7-kb genome, and the 0.8-kb antigenomic-sense RNA. The former two RNA species form circular RNA and represent the replication products of the HDV RNA genome. The 0.8-kb RNA, however, is polyadenylated and thus resembles cellular pol II transcripts. This RNA is the mRNA for translation of HDAg (29). In the HDV-infected cells, two forms of HDAg are found: a small form (S-HDAg), which is 195 amino acids in length, and a

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large form (L-HDAg), which is 214 amino acids in length (2, 4, 35, 38). Both forms are translated from the same open reading frame present on the 0.8-kb mRNA; the large form results from an RNA editing event (5, 36, 37), extending the S-HDAg open reading frame by 19 amino acids to encode the L-HDAg. The S-HDAg is required for HDV RNA replication *in vivo* (26). In contrast, the L-HDAg inhibits HDV RNA replication (7, 17).

Since the 1.7-kb antigenome and the 0.8-kb mRNA are synthesized from the same genomic RNA template, a central question is how the synthesis of these two RNA species is regulated. An earlier hypothesis based on the results from cDNA transfection studies proposed that the primary transcript from the genomic RNA template is the 0.8-kb mRNA (8, 18). Synthesis of the 1.7-kb antigenome occurs only after suppression of the polyadenylation signal by HDAg (19, 20). In this model, the same cellular polymerase is proposed to carry out the synthesis of both the 1.7-kb antigenome and 0.8-kb mRNA. However, using a cDNA-free transfection system (33), we found that the 0.8-kb mRNA continues to be synthesized throughout the replication cycle, and increasing amounts of HDAg do not suppress the synthesis of the 0.8-kb mRNA. These findings suggested that the synthesis of the 1.7-kb antigenome and the synthesis of the 0.8-kb mRNA are under different mechanisms of regulation.

We have investigated the enzymatic requirements of HDV RNA replication (synthesis of the 1.7-kb antigenome) and transcription (synthesis of the 0.8-kb mRNA) from the HDV genomic RNA template in cell culture. Using a cDNA-free HDV RNA transfection system, we established that the synthesis of the 0.8-kb mRNA is sensitive to α -amanitin at a concentration of 3 μ g/ml and this inhibition can be relieved by an α -amanitin-resistant pol II mutant. Surprisingly, synthesis of the 1.7-kb antigenome is insensitive to α -amanitin at concentrations as high as 25 μ g/ml. Furthermore, the synthesis of the 1.7-kb antigenome, but not the 0.8-kb mRNA, is dependent on the production of newly translated S-HDAg, further discriminating between the mechanisms of synthesis of these two RNA species. Finally, synthesis of the 0.8-kb mRNA from the HDV RNA genome can be distinguished from DNA-templated mRNA synthesis by cellular pol II. Taken together, these results support the hypotheses that pol II, or at least protein products from pol II transcripts, is involved in the synthesis of the 0.8-kb mRNA from the HDV RNA genome and that the mechanisms of synthesis of the 1.7-kb antigenome and the 0.8-kb mRNA are distinct. Furthermore, they raised the interesting possibility that the 1.7-kb HDV antigenomic RNA is synthesized by a cellular polymerase other than pol II.

MATERIALS AND METHODS

Cell culture and transfection. Huh7 cells (34) were cultured at 37°C in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU of penicillin per ml, 100 mg of streptomycin per ml, 2 mM L-glutamate, and 1% nonessential amino acids (complete DMEM). BC10 M/E cells, a mouse cell line (10), were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum, 100 IU of penicillin per ml, and 100 mg of streptomycin per ml. The BC10ME-derived permanent cell line BCHAWT, which expresses an α -amanitin-resistant mutant pol II, was selected in G418 at 600 μ g/ml after transfection with plasmid pHAWT (see below). The resulting clones were further selected in α -amanitin (Sigma) at 10 μ g/ml, and the clones resistant to both G418 and α -amanitin were expanded for further analysis. E10 control cells transfected with the empty vector pcDNA3 were selected in G418 only. E10 and BCHAWT cells were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum, 100 IU of penicillin per ml, and 7.5 μ g of gentamicin per ml. Ts63 cells, which were derived from a temperature-sensitive hamster cell line (42) and stably express S-HDAg from an integrated cDNA copy of the HDAg-encoding mRNA under the control of the cytomegalovirus (CMV) promoter (22), were cultured at 34°C in DMEM supplemented with 10% fetal bovine serum, 100 IU of penicillin per ml, and 7.5 μ g of gentamicin per ml. All transfections were performed using

the DMRIE-C reagent (Gibco BRL) in accordance with the protocol provided by the manufacturer, with slight modifications. Briefly, 1 day prior to transfection, cells were seeded onto 60-mm-diameter dishes. On the following day, cells were transfected with an appropriate amount of RNA (typically 5 to 10 μ g) in 2 ml of transfection mixture in serum-free medium. After 1 to 2 h, 2 ml of culture medium containing 20% fetal bovine serum was added to the cells. Following incubation overnight, the culture medium was replaced with fresh medium and the cells were further incubated for an additional 1 to 5 days. For experiments involving the use of α -amanitin, various amounts of α -amanitin dissolved in sterile water were added directly to the culture medium.

Vectors and plasmid construction. Construction of plasmid PX9-I/II, which expresses the genotype I/II chimeric S-HDAg, was reported previously (33). Plasmid pKS/HDV1.9, which expresses 1.9-kb genomic-sense HDV RNA (a full-length HDV genome plus 200 additional HDV nucleotides [nt]) under the control of the CMV promoter, and pKS/H2ag, which expresses the antigenomic strand of a mutant trimer HDV RNA under the control of the CMV promoter, were described in a previous study (24). pCMV/neo-Sm (25), which expresses an S-HDAg, was used for HDV cDNA transfection experiments. Plasmid pKS/HDV1.9m, which expresses 1.9-kb genomic-sense HDV RNA with a truncated open reading frame for HDAg, was constructed by digesting pKS/HDV1.9 (24) with *Afl*III (nt 1209), followed by a fill-in reaction with the Klenow fragment to blunt the ends. The blunt-ended product was self-ligated to produce the final plasmid, which contains an insertion of 5 nt to introduce a stop codon. Plasmid pBS/T7GSacII, which expresses nt 25 to 658 of the HDV genomic-sense RNA under the control of the T7 promoter, was constructed by deleting the *Sac*II fragment from plasmid pBS/T7G, which contains the *Pst*I fragment of the American HDV isolate (32) inserted into the *Pst*I site of pBSII/KS+. Plasmid pArg-Maxi, used to measure pol III transcription, is a derivative of a *Drosophila* tRNA^{Arg} gene where 12 nt have been inserted between the internal promoter regions to serve as a marker (15). Plasmid pHAWT, which contains the mouse genomic DNA encoding an α -amanitin-resistant mutant form of the pol II largest subunit, RPII215, with a 793N→D mutation (1), was a gift from J. L. Corden (Johns Hopkins University, Baltimore, Md.). A hemagglutinin tag was placed at the N terminus of the protein.

In vitro transcription. Genomic HDV RNA (1.9 kb) which contains the entire HDV genome plus approximately 200 additional nt of the HDV sequence was transcribed from pKS/HDV1.9 (24) using the T7 MEGAscript transcription kit (Ambion) after linearization of the plasmid by *Eco*RV digestion. Antigenomic HDV RNA (1.9 kb) was transcribed from pKS/HDV1.9 using SP6 MEGAscript (Ambion) after linearization of the plasmid by *Sna*BI digestion. Capped mRNAs used for translation of wild-type and genotype I/II-chimeric S-HDAg were transcribed from plasmids PX9 (33) and PX9-I/II, respectively, using T7 mMES-SAGE mMACHINE (Ambion) after linearization of plasmids by *Hind*III digestion.

Northern blot analysis. Total RNA was extracted from transfected cells using the guanidinium thiocyanate method (9). The RNA was digested with RQ1 DNase (Promega), treated with formaldehyde, electrophoresed through formaldehyde-containing 1.2% agarose gels, blotted onto a nitrocellulose membrane (Hybond C extra; Amersham), and probed with [³²P]UTP-labeled HDV strand-specific riboprobes. Riboprobes were transcribed from plasmid S18 (32) (to detect genomic HDV RNA) after linearization with *Hind*III digestion or from pBS/T7GSacII (to detect antigenomic HDV RNA in the noncoding region) after linearization of plasmids with *Hind*III digestion. To detect newly synthesized 0.8-kb mRNA, ³²P-end-labeled oligonucleotide 1565A (1565-CCCCGCGGTCTTTCCTCTTTTCGGACC-1581) (33), which is specific for the American isolate of genotype I HDV (32), was used as a probe. The protocol for Northern blots using oligonucleotide probes was adapted from a published protocol (16).

Western blot analysis. Protein was extracted from transfected cells by the standard method (39) and separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond C extra; Amersham) and detected by the ECL Western blot detection system (Amersham) using a combination of three monoclonal antibodies against HDAg (23) and visualized by autoradiography.

RNAse protection assay. RNA was analyzed by RNAse protection assay using an RPA II RNAse Protection Assay Kit (Ambion). Briefly, [³²P]UTP-labeled probe (antisense to the expressed Arg maxigene) was transcribed from plasmid pArg-Maxi after linearization with *Xba*I digestion. Approximately 10⁶ cpm of radiolabeled probe was mixed with 2.5 μ g of total RNA from the transfected cells, heat denatured, and incubated at 43°C overnight. After hybridization, the sample was digested by RNAse A and T₁ and precipitated in accordance with the protocol provided with the kit. The precipitated RNA was resuspended in 8 μ l of gel loading buffer (provided with the kit) and subjected to electrophoresis at 20 mA in an 8% acrylamide–8 M urea gel. The protected bands were visualized by autoradiography.

RESULTS

Synthesis of the 0.8-kb mRNA is sensitive to inhibition by a low concentration of α -amanitin in cell culture. In order to evaluate the possible role of pol II in HDV RNA replication, we examined the sensitivity of HDV RNA synthesis to α -ama-

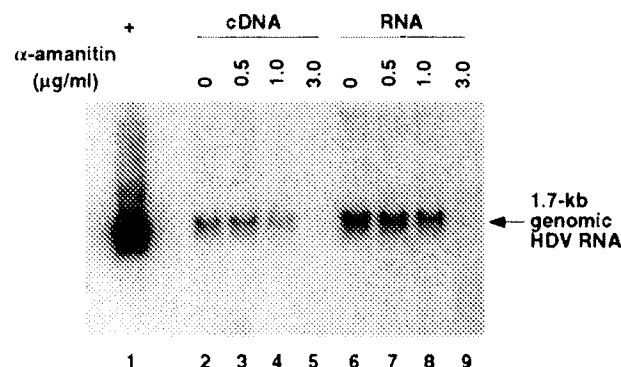


FIG. 1. Northern blot demonstrating the effects of α -amanitin on HDV genomic RNA synthesis from either cDNA or RNA templates. Huh7 cells were transfected with either plasmid pKS/1.9 or in vitro-transcribed 1.9-kb antigenomic HDV RNA plus an HDAg-encoding mRNA (33). The indicated amounts of α -amanitin were added to cells at the time of transfection. Cells were harvested 3 days posttransfection, and total RNA was analyzed by Northern blot assay using 32 P-labeled antigenomic-sense HDV RNA as a probe. Lanes: 1, total RNA from H189 cells indicating the position of the 1.7-kb genomic RNA; 2 to 5, total RNA from cells transfected with HDV cDNA; 6 to 9, total RNA from cells transfected with HDV RNA.

nitin in cell culture. Previous studies on this issue have used in vitro nuclear lysates that contained an HDV cDNA template and thus were compromised by an artificial requirement for DNA-templated transcription. Furthermore, these assays were not efficient enough for identification of different HDV RNA species (14, 31). To circumvent these problems, we used a recently established HDV RNA transfection approach (33) in which Huh7 cells were transfected with in vitro-transcribed antigenomic-sense 1.9-kb HDV RNA and capped mRNA encoding S-HDAg. As a comparison, we also performed HDV cDNA transfection using a plasmid that would transcribe a 1.9-kb genomic-sense HDV RNA under the control of the CMV promoter. The primary transcripts from both the transfected RNA and DNA are genomic-sense HDV RNAs, and both approaches lead to robust HDV RNA replication. Various concentrations of α -amanitin were added to the culture at the time of transfection, and HDV genomic-sense RNA was examined 3 days posttransfection. We found that Huh7 cells could survive for at least 3 days in the presence of α -amanitin, although the cells showed some cytotoxicity after 2 days of treatment (data not shown). As shown in Fig. 1, HDV RNA (1.7-kb) synthesis in both RNA- and DNA-transfected cells was inhibited by α -amanitin at 3 μ g/ml consistent with the previous in vitro data (31) and with the hypothesis that pol II mediates both RNA- and DNA-templated HDV RNA synthesis. Similar results were obtained when the reverse experiment was performed, i.e., the in vitro-transcribed 1.9-kb genomic-sense RNA was used for transfection and antigenomic RNA synthesized in the transfected cells was examined (data not shown).

Since α -amanitin was added at the time of transfection, the inhibition of HDV RNA (1.7 kb) replication by α -amanitin observed in this experiment could be an indirect effect of inhibition of 0.8-kb mRNA transcription if a large amount of HDAg needs to be synthesized before HDV RNA replication can occur. Also, the inhibition of HDV RNA synthesis could be a nonspecific cytotoxic effect of prolonged treatment with α -amanitin. To examine these possibilities, we performed another experiment in which α -amanitin was not added until 3 days posttransfection to allow a sufficient amount of HDAg to accumulate prior to α -amanitin treatment. HDV antigenomic-

sense RNA was analyzed on day 4 to detect both the 1.7-kb and 0.8-kb RNA species (Fig. 2). The result showed that the synthesis of the 0.8-kb mRNA was completely inhibited by α -amanitin at 5 μ g/ml, suggesting that α -amanitin inhibits HDV subgenomic mRNA synthesis per se. Surprisingly, under this condition, the synthesis of the 1.7-kb antigenomic RNA was almost completely resistant to α -amanitin at a concentration as high as 25 μ g/ml. α -Amanitin at 100 μ g/ml caused a slight decrease in 1.7-kb RNA. The mechanism of resistance of this RNA species to α -amanitin was further investigated, and the findings are presented in later sections.

As a control, *choA* RNA, which is an endogenous cellular pol II transcript, was found to be inhibited by a low concentration of α -amanitin, although *choA* RNA appears to be more resistant than the 0.8-kb HDV RNA, probably because of the longer half-life of *choA* mRNA. These results suggest that transcription of the 0.8-kb HDV mRNA is at least as sensitive to α -amanitin as the cellular pol II transcripts.

To further rule out the possibility that α -amanitin caused generalized inhibition of transcription, we tested the transcription from an RNA pol III-dependent promoter under the same condition. HDV RNA and a pol III reporter plasmid, pArg-Maxi (15), which expresses tRNA^{Arg} containing a 12-nt insertion to allow specific detection of this transcript, were cotransfected into Huh7 cells. We used a higher concentration of α -amanitin for this experiment than necessary for inhibition of the 0.8-kb mRNA. Cells were treated with α -amanitin (20 μ g/ml) on days 1 and day 2 posttransfection, and RNA was harvested 1 day later. The results showed that, at both time points, the synthesis of the 0.8-kb HDV mRNA was completely inhibited (Fig. 3A, lanes 2 to 5); in contrast, transcription from the pol III promoter, as detected by RNase protection assay, was not affected by α -amanitin at all (Fig. 3B, lanes 1 to 4). This result supports the conclusion that the inhibition of 0.8-kb HDV mRNA synthesis in α -amanitin-treated cells was due to specific inhibition of pol II transcription but was not a result of the generalized cytotoxicity of α -amanitin. This result also

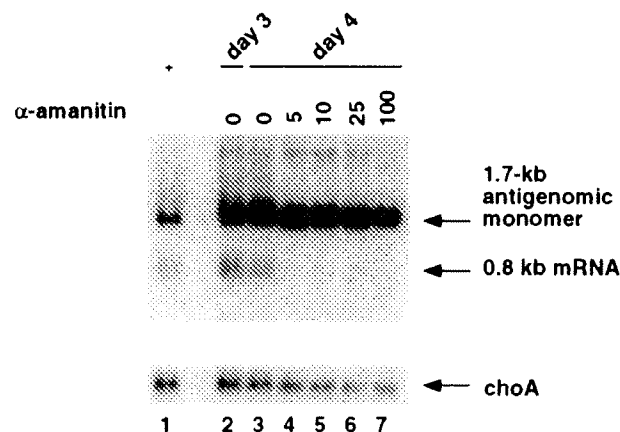


FIG. 2. Sensitivity of 0.8-kb mRNA synthesis to α -amanitin. Huh7 cells were cotransfected with in vitro-transcribed 1.9-kb genomic RNA and 0.8-kb HDV mRNA (33). Cells were treated with different concentrations of α -amanitin on day 3 posttransfection, and RNA was harvested on day 4. The RNA blot was probed for HDV antigenomic-sense RNA with 32 P-end-labeled oligonucleotide 1565A, which detects the mRNA transcribed from the 1.9-kb genomic RNA but not the transfected mRNA (33). The same membrane was probed for *choA* mRNA. Lanes: 1, positive control from previously transfected Huh7 cells indicating the positions of the 1.7-kb antigenome and the 0.8-kb HDV mRNA; 2, total RNA from transfected cells on day 3 posttransfection; 3 to 7, total RNA from transfected cells at day 4 posttransfection after treatment with the indicated amounts of α -amanitin on day 3.

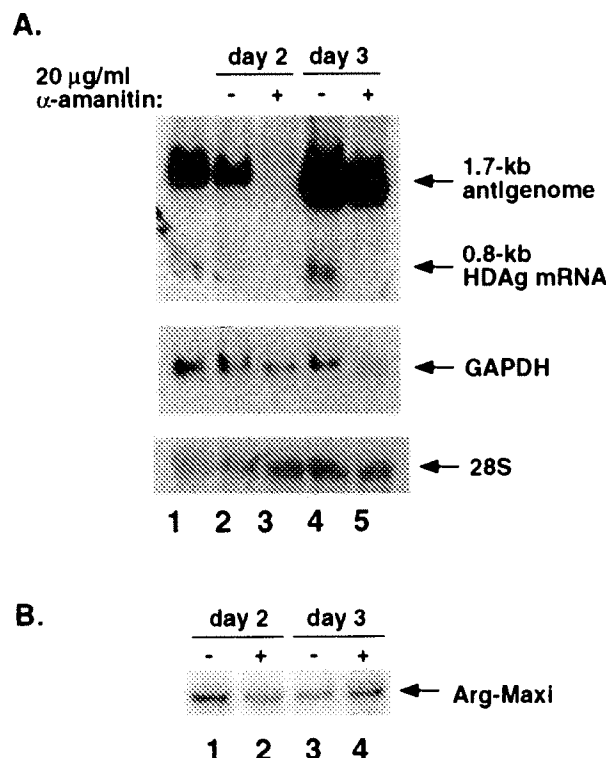


FIG. 3. Comparison of the effects of α -amanitin on the synthesis of various RNA species. Huh7 cells were transfected with in vitro-transcribed 1.9-kb HDV genomic RNA, 0.8-kb HDV mRNA, and pArg-Maxi (under the control of a pol III promoter). Cells were treated with 20 μ g of α -amanitin per ml on either day 1 or day 2 posttransfection, and cells were harvested 1 day after α -amanitin treatment. Samples were analyzed by either Northern blot assay (A) to detect antigenomic-sense HDV RNA (upper panel), GAPDH (middle panel), and 28S rRNA (bottom panel) or by RNase protection assay (B) to detect Arg-Maxi tRNA. (A) Antigenomic HDV RNA was detected with 32 P-end-labeled oligonucleotide 1565A. The same membrane was probed for GAPDH mRNA and 28S rRNA. Lanes: 1, RNA size markers for the 1.7-kb antigenome and the 0.8-kb mRNA; 2 and 3 and 4 and 5, total RNA from transfected cells on days 2 and 3, respectively, with or without α -amanitin treatment (added 24 h prior to harvest). (B) RNase protection assay of RNA samples in panel A using 32 P-labeled antisense Arg-Maxi as a probe. Lanes 1 to 4 correspond to lanes 2 to 5 in panel A.

showed that the 1.7-kb antigenomic RNA was resistant to α -amanitin treatment when α -amanitin was added on day 2 and RNA was examined on day 3 (Fig. 3A, lanes 4 and 5). However, when α -amanitin was added on day 1 and RNA was examined 1 day later, the synthesis of the 1.7-kb antigenome was completely inhibited by α -amanitin (Fig. 3A, lanes 2 and 3). These results confirmed those presented in Fig. 1 and 2. As a comparison, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was inhibited by α -amanitin to the same extent, regardless of when α -amanitin was added. Under the same conditions, the synthesis of 28S rRNA was not inhibited.

It should be noted that the amount of the 1.7-kb RNA found on day 3 in the presence of α -amanitin (Fig. 3A, lane 5) was significantly higher than that found on day 2, even in the absence of α -amanitin (lane 2), suggesting that RNA synthesis continued to take place in the presence of α -amanitin. Therefore, the insensitivity of the 1.7-kb RNA to α -amanitin represents the true resistance of its synthesis to α -amanitin treatment and not to stabilization of the RNA. These findings established that the synthesis of the 0.8-kb mRNA is specifically inhibited by the low concentration of α -amanitin, supporting the hypothesis that 0.8-kb mRNA synthesis is pol II

dependent. In contrast, the synthesis of the 1.7-kb antigenomic RNA is not sensitive to α -amanitin unless α -amanitin is added early.

Synthesis of the 1.7-kb antigenomic RNA is insensitive to inhibition by α -amanitin after abundant S-HDAg appears. The relative resistance of 1.7-kb antigenomic RNA synthesis to α -amanitin after day 2 or 3 posttransfection was further studied. First, we re-evaluated the data shown in Fig. 2 to focus on the 1.7-kb RNA species. Shorter exposure of the membrane in Fig. 2 demonstrated that the level of the 1.7-kb antigenomic RNA continued to increase from day 3 to day 4, even in the presence of as much as 25 μ g of α -amanitin per ml (Fig. 4, compare lanes 4 to 7 with lane 2). This result clearly indicated that α -amanitin does not inhibit the synthesis of 1.7-kb antigenomic RNA. In contrast, the amount of *choA* RNA decreased in the presence of α -amanitin. The level of the 1.7-kb antigenomic RNA was decreased slightly by α -amanitin at 100 μ g/ml. These results suggest that the synthesis of the 1.7-kb antigenomic HDV RNA does not require pol II and that the observed sensitivity of the 1.7-kb RNA to α -amanitin treatment during days 1 and 2 after transfection (Fig. 3A) is likely an indirect effect of the inhibition of 0.8-kb mRNA synthesis by α -amanitin.

To more carefully establish the time-related effects of α -amanitin on 1.7-kb antigenomic HDV RNA synthesis, α -amanitin was added at several time points after HDV RNA transfection and RNA was examined 2 or 3 days later (Fig. 5A). The results showed that when α -amanitin (5 μ g/ml) was added on day 1 and RNA was examined on day 3, the 1.7-kb HDV RNA was not detected (lane 3). When α -amanitin was added on day 2, again virtually no 1.7-kb RNA was detected on day 4 (lane 5). However, when it was added on day 3 and RNA was examined on day 6, the amount of 1.7-kb RNA did not decrease but, in fact, continued to increase (lane 7). The finding that the amount of 1.7-kb RNA actually decreased when α -amanitin was added during the first 2 days indicates that RNA synthesis was indeed inhibited and that the RNA was not unduly stable. Furthermore, the amount of HDV RNA actually increased from day 3 to day 6 (compare lanes 2 and 7), even in the presence of α -amanitin. Therefore, the resistance of 1.7-kb RNA to α -amanitin at later time points represents true resistance of the RNA synthesis and was not due to stabilization of RNA. It should be noted that the timing of the switch of the 1.7-kb RNA from sensitivity to resistance to α -amanitin coincides with the appearance of abundant S-HDAg in HDV RNA-transfected cells on day 3 posttransfection (33) (data not shown), suggesting that 1.7-kb antigenomic RNA synthesis is not inhibited by α -amanitin if S-HDAg is present. *choA* RNA

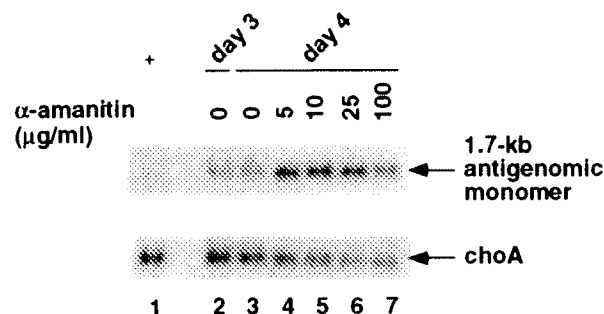


FIG. 4. Northern blot analysis of effects of α -amanitin on antigenomic RNA synthesis on day 3 posttransfection. The blot is the same as that in Fig. 2 but with a shorter exposure time to clearly visualize the 1.7-kb antigenomic RNA. Lanes are identical to those described in the legend to Fig. 2.

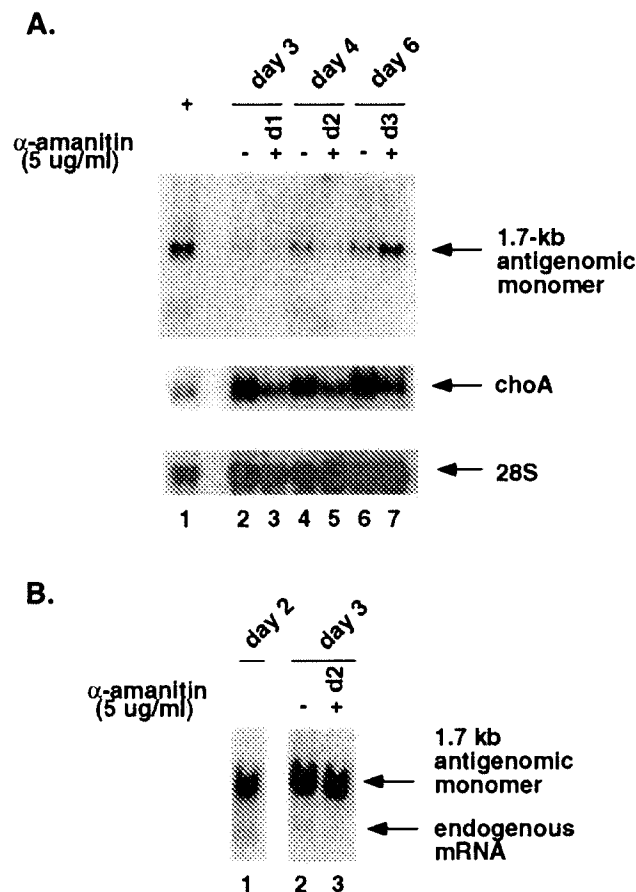


FIG. 5. (A) Sensitivity of various RNA species to α -amanitin at different time points after HDV RNA transfection. Huh7 cells were transfected with 1.9-kb genomic RNA and 0.8-kb mRNA and treated with α -amanitin (5 μ g/ml) at different time points. The RNA blot was probed for the different RNA species. Lanes: 1, RNA size markers; 2, 4, and 6, total RNA harvested at days 3, 4, and 6 from transfected cells without α -amanitin treatment; 3, 5, and 7, total RNA harvested at days 3, 4, and 6 from transfected cells treated with α -amanitin at days 1, 2, and 3, respectively. (B) Northern blot analysis of total RNA harvested from Ts83 cells transfected with in vitro-transcribed 1.9-kb HDV genomic HDV RNA only. Cells were treated with 5 μ g of α -amanitin per ml on day 2 post-transfection and harvested on day 3. Lanes: 1, RNA from untreated cells harvested on day 2; 2, RNA from untreated cells harvested on day 3; 3, RNA from cells treated with α -amanitin on day 2 and harvested on day 3.

was inhibited to the same extent by α -amanitin at all three time points, whereas 28S rRNA was not inhibited.

To prove that 1.7-kb RNA synthesis is not inhibited by α -amanitin if abundant S-HDAg is present in the cells, we next examined the α -amanitin sensitivity of HDV RNA synthesis in Ts83 cells (22), which constitutively express S-HDAg, after HDV RNA transfection (Fig. 5B). The results showed that the synthesis of the 1.7-kb antigenomic RNA was not inhibited when α -amanitin was added on day 2 posttransfection; in contrast, the endogenous HDAg mRNA (1.1 kb), which was expressed from an integrated cDNA encoding HDAg (22, 33), was inhibited. This result provides further evidence that 1.7-kb antigenomic RNA synthesis is not inhibited by α -amanitin and that its inhibition at early time points is secondary to inhibition of S-HDAg production.

The results above showed that the amount of 1.7-kb HDV RNA continued to increase even in the presence of α -amanitin, indicating that HDV RNA synthesis was not inhibited by

α -amanitin. Furthermore, 1.7-kb antigenomic RNA was less stable than the *choA* mRNA. To further demonstrate that the 1.7-kb HDV RNA was not unusually stable, we examined the stability of the transcript from a plasmid, pKS/H2ag, which transcribes a mutant antigenomic HDV trimer RNA from the CMV promoter (24). The HDV sequence in this plasmid contains a mutation in the ribozyme domain of the genomic strand of HDV RNA, such that the antigenomic RNA transcribed from the plasmid can be processed into 1.7-kb RNA but cannot be replicated (24). Thus, the 1.7-kb antigenomic RNA detected in the transfected cells is derived exclusively from the primary transcript of the HDV cDNA. The synthesis of this transcript from DNA can be inhibited by α -amanitin at any time point, thus allowing measurement of the stability of the 1.7-kb antigenomic RNA. Previous studies have shown that the HDV RNA transcript from this plasmid consists of both linear and circular RNA species and that the RNA transcript derived from the corresponding wild-type plasmid leads to robust RNA replication (24), indicating that the RNA transcript represents the natural HDV RNA species. Huh7 cells were transfected with pKS/H2ag and pCMV/neo-Sm, which expresses a wild-type S-HDAg, mimicking the conditions of the RNA-based transfection studies. Three days after transfection, 5 μ g of α -amanitin per ml was added to cells and total RNA was harvested at different time points. RNA was analyzed under conditions in which the linear and circular RNAs were separable (24). The results showed that after 1 day of α -amanitin treatment, the primary transcript (5.1-kb antigenomic trimer) became undetectable. The linear form of 1.7-kb or 3.4-kb RNA was significantly reduced within 1 day of α -amanitin treatment (Fig. 6, compare lanes 1 and 2). The circular form appeared to be more stable than the linear form but is nonetheless not more stable than the *choA* mRNA (also Fig. 2 and 4). Therefore, the finding that the amount of 1.7-kb antigenomic RNA

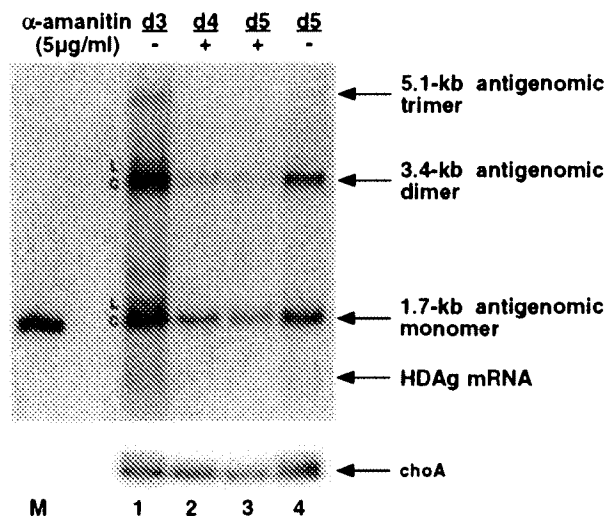


FIG. 6. Comparison of the stability of HDV antigenomic RNA and *choA* mRNA. Huh7 cells were transfected with plasmids pKS/H2ag and pKS/CMV-sm (see text). Cells were treated with α -amanitin at 5 μ g/ml on day 3 posttransfection. Total RNA was harvested at various time points after treatment. RNA was separated by electrophoresis on 1.5% agarose gels as previously described (24, 25). Samples were analyzed by Northern blot, using first a 32 P-labeled HDV-specific probe and then a *choA* probe. Lanes: 1 and 4, total RNA from transfected cells harvested on days 3 and 5, respectively, without α -amanitin treatment; 2 and 3, total RNA from transfected cells to which α -amanitin was added on day 3, harvested on days 4 and 5, respectively. L, linear RNA; C, circular RNA; M, monomer RNA marker.

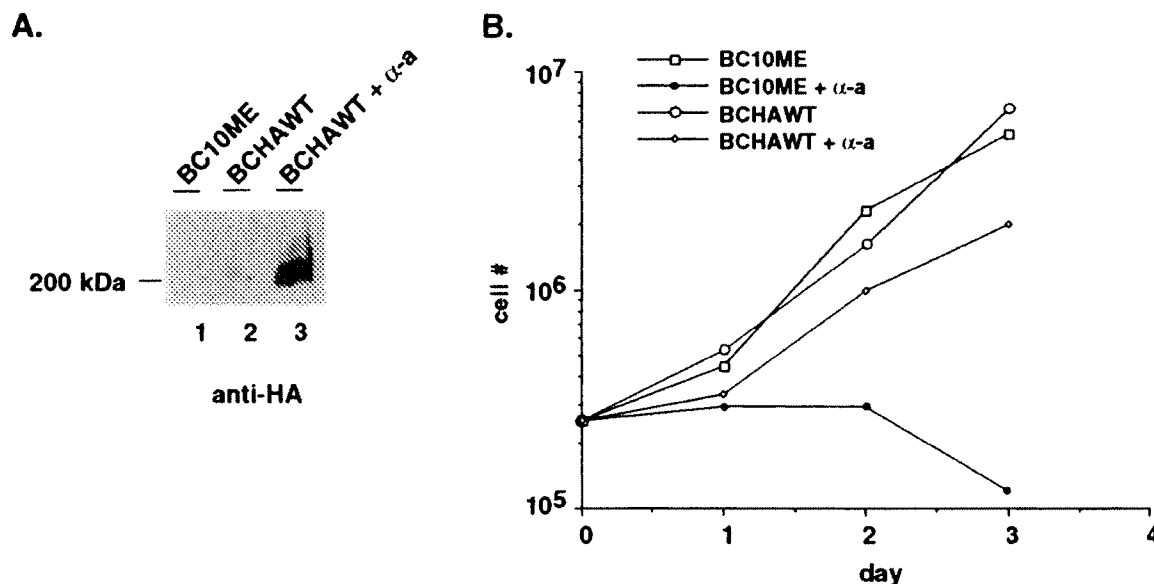


FIG. 7. Characterization of the cell line BCHAWT, which expresses α -amanitin-resistant mutant pol II. (A) Western blot analysis of the mutant pol II in BCHAWT cells. Cells were treated with α -amanitin (5 μ g/ml) for 2 days and harvested for Western blotting using an anti-hemagglutinin monoclonal antibody as a probe. Lanes: 1, BC10ME cells; 2, untreated BCHAWT cells; 3, BCHAWT cells treated with α -amanitin for 2 days. (B) Growth kinetics. Culture dishes (60-mm diameter) were seeded with 10^5 BC10ME or BCHAWT cells and cultured in the presence or absence of α -amanitin at 5 μ g/ml. Cells were counted with a hemacytometer on days 1, 2, and 3 after seeding.

was not reduced by α -amanitin treatment most likely reflects the true resistance of the synthesis of this RNA to α -amanitin.

An α -amanitin-resistant pol II mutant partially restores transcription of the 0.8-kb mRNA. To further determine if the inhibition of 0.8-kb mRNA synthesis by α -amanitin can be attributed to the inhibition of pol II activity, we examined whether transcription of this mRNA in the presence of α -amanitin could be restored in cells expressing an α -amanitin-resistant pol II mutant. We first established a cell line (BCHAWT) expressing α -amanitin-resistant mutant pol II (1). The expression of this transfected mutant pol II in this cell line was very low but was significantly induced after 2 days of α -amanitin treatment (Fig. 7A). This cell line continued to grow in the presence of α -amanitin at 5 μ g/ml but had a slightly slower growth rate than in the absence of α -amanitin, probably due to the slow induction of the mutant pol II. In contrast, the parental cell line (BC10ME) failed to grow in the presence of α -amanitin (Fig. 7B).

This cell line was used to study the effect of α -amanitin on 0.8-kb mRNA synthesis after HDV RNA transfection. In the absence of α -amanitin, HDV RNA replication and mRNA synthesis in this cell line were only slightly less robust than in the control cells that were transfected with the vector alone (data not shown). To fully induce the α -amanitin-resistant pol II, cells were treated with α -amanitin at the time of HDV RNA transfection and newly synthesized HDV antigenomic-sense RNA was examined on days 3 and 4. The results showed that *choA* RNA transcription in this cell line was not affected by α -amanitin at up to 25 μ g/ml, indicating that the mutant pol II conferred resistance to α -amanitin (Fig. 8, bottom). The 0.8-kb mRNA also could be detected even with α -amanitin at 25 μ g/ml; however, the overall levels of this RNA were lower than in untreated cells (Fig. 8, top). These results indicate that the synthesis of this mRNA was partially restored by the α -amanitin-resistant mutant pol II. Therefore, we conclude that the 0.8-kb HDV mRNA synthesis is mediated by pol II or requires

protein products of pol II transcripts; however, HDV mRNA synthesis appears to require more factors than are involved in the transcription of cellular genes, since the α -amanitin-resistant mutant pol II fully restored *choA* RNA synthesis but only partially restored 0.8-kb HDV mRNA synthesis. Significantly, the 1.7-kb antigenomic RNA was not inhibited by α -amanitin in this cell line, even when α -amanitin was added at the time of HDV RNA transfection. As shown above, this was likely the result of restoration of 0.8-kb mRNA synthesis.

Temperature dependence of 0.8-kb mRNA synthesis from the HDV RNA template. To further distinguish RNA-templated from DNA-templated pol II transcription, we examined these two types of transcription at different temperatures. We had previously found that HDV RNA replicated much better

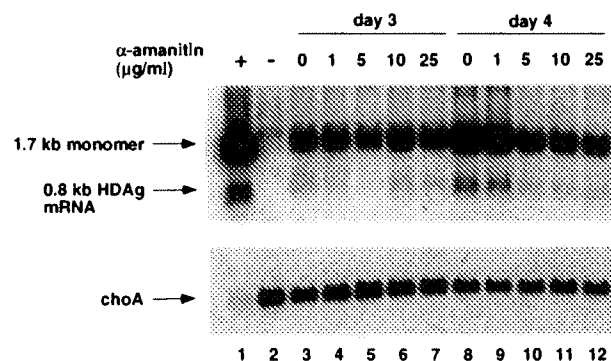


FIG. 8. Effects of α -amanitin on HDV RNA synthesis in BCHAWT cells. BCHAWT cells were transfected with in vitro-transcribed 1.9-kb genomic HDV RNA and 0.8-kb mRNA. Various amounts of α -amanitin were added immediately after transfection. Total RNA was harvested on days 3 and 4 posttransfection, and HDV antigenomic RNA and *choA* mRNA were detected by Northern blot assay as described in the legend to Fig. 2. Lanes: 1, RNA size markers; 2, untransfected BCHAWT cells.

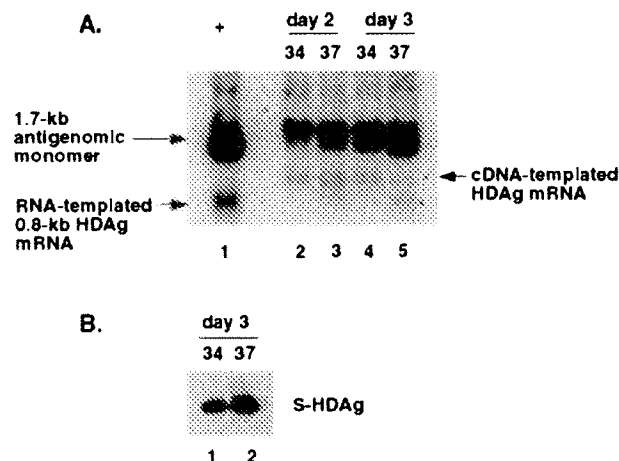


FIG. 9. HDV RNA replication in Ts83 cells at different temperatures. Ts83 cells were transfected with in vitro-transcribed 1.9-kb genomic HDV RNA only and maintained at 34 or 37°C. Total RNA was harvested on days 2 and 3, and protein was harvested on day 3. (A) Northern blot detecting antigenomic HDV RNA. Lane 1, RNA size markers. (B) Western blot analysis of HDV RNA-transfected Ts83 cells grown at 34 and 37°C.

at 37°C than at 34°C, whereas cellular mRNA synthesis was equivalent at both temperatures (22). Further, we had found that when Ts83 cells grown at 34°C were transfected with HDV RNA, only the HDV mRNA transcribed from the integrated HDV cDNA, but not the 0.8-kb mRNA synthesized from the transfected HDV RNA, could be detected (33). To establish the differential temperature sensitivity of RNA- versus DNA-templated mRNA transcription, we further studied HDV mRNA synthesis in Ts83 cells transfected with HDV RNA at 34°C versus 37°C (Fig. 9A). The results showed that the subgenomic mRNA transcribed from the integrated HDV cDNA, which is 1.1 kb in length (33), was detectable at both 34 and 37°C; in contrast, the 0.8-kb mRNA transcribed from the transfected RNA was detected only at 37°C. The 1.7-kb HDV RNA was also more abundant at 37°C than at 34°C, which could be the result of increased production of HDV at 37°C (Fig. 9B). These results further demonstrate that RNA-templated transcription of the HDV mRNA has properties distinct from those of DNA-templated synthesis of a similar transcript.

Transcription of the 0.8-kb HDV mRNA can occur in the absence of HDV RNA replication. The data shown above indicated that replication of HDV genomic-length RNA and transcription of 0.8-kb mRNA may involve different polymerases and have different metabolic requirements. Furthermore, 1.7-kb HDV RNA synthesis can occur in the absence of 0.8-kb mRNA transcription, once a sufficient amount of HDV is made. We next examined whether the reverse is also true, i.e., whether 0.8-kb mRNA transcription can occur in the absence of the 1.7-kb RNA synthesis. A previous report showed that an HDV genomic RNA encoding a defective S-HDAg could not replicate even when it was transfected together with a wild-type S-HDAg as a ribonucleoprotein complex (13). We have also found that such a defective HDV RNA could not replicate even when it was transfected together with an in vitro-transcribed mRNA encoding the wild-type S-HDAg (data not shown). We therefore used this approach to determine whether 0.8-kb mRNA transcription could occur in the absence of HDV RNA replication.

Since the amount of HDV mRNA made, if any, in the absence of HDV RNA replication is expected to be small because the amount of template RNA will be very limited, we

designed an experimental approach to increase the sensitivity of detection of HDV mRNA. For this purpose, we used a mutant HDV genomic RNA that encodes a truncated HDV Ag. Since this protein can be translated only from the 0.8-kb mRNA (29), the accumulation of the truncated HDV Ag indicates the production of the HDV mRNA. Huh7 cells were transfected with in vitro-transcribed S-HDAg-encoding mRNA and either the wild-type or the mutant 1.9-kb genomic HDV RNA in accordance with the previously reported protocol (33). Western blot analysis of protein from cells harvested on day 2 posttransfection showed that cells transfected with these RNAs produced comparable levels of the wild-type and truncated (m-HDAg) HDV Ags, respectively (Fig. 10A). Since m-HDAg can be synthesized only from the subgenomic mRNA (29), this result suggests that the 0.8-kb mRNA was transcribed from the mutant HDV genome.

This conclusion was confirmed by Northern blot analysis of

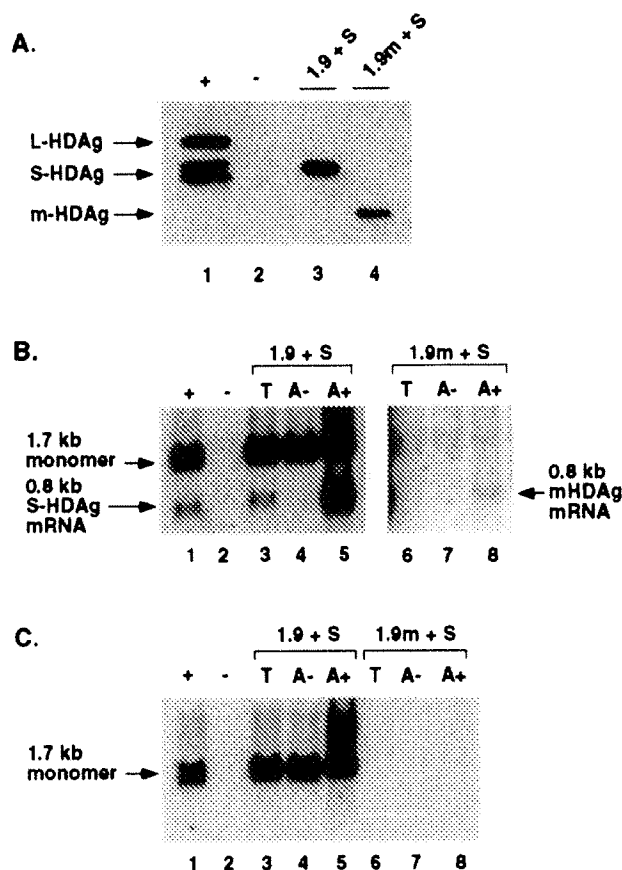


FIG. 10. Analysis of 0.8-kb mRNA in the absence of HDV genomic RNA synthesis. Huh7 cells were transfected with in vitro-transcribed 0.8-kb mRNA and either wild-type or mutant 1.9-kb genomic HDV RNA. Cells were harvested 2 days after transfection and analyzed by Western blot assay for HDV Ag (A) and by Northern blot assay for HDV-specific RNA (B and C). (A) Western blot. Lanes: 1, Huh7 cells transfected with plasmids encoding S- and L-HDAg to serve as protein markers; 2, untransfected cells; 3 and 4, cells transfected with in vitro-transcribed S-HDAg mRNA (S) and either wild-type (lane 3) or mutant (lane 4) 1.9-kb genomic HDV RNA. (B) Northern blot probed with oligonucleotide 1565A. Lanes: 1, RNA size markers; 2, total RNA from untransfected cells; 3 to 5, total (T) poly(A)⁺, and poly(A)⁺ RNAs from Huh7 cells transfected with wild-type 1.9-kb genomic HDV RNA and S-HDAg mRNA; 6 to 8, corresponding RNAs from cells transfected with mutant 1.9-kb genomic HDV RNA and S-HDAg mRNA. Lanes 1 to 5 were exposed to autoradiography for 24 h, while lanes 6 to 8 were exposed for 48 h. (C) The same blot as in panel B probed with an HDV RNA detecting antigenomic RNA in the noncoding region of the genome.

the poly(A)-enriched RNA from transfected cells (Fig. 10B); a substantial amount of the 0.8-kb mRNA was made from the wild-type genomic HDV RNA, whereas a small amount was also detected in cells transfected with the mutant genomic HDV RNA. This RNA species was not detected in the total RNA fraction of the mutant RNA-transfected cells, indicating that only a small amount of the mRNA was made. Because of cross-hybridization of the oligonucleotide probe to 18S rRNA, the status of the 1.7-kb HDV RNA in cells transfected with the mutant HDV genome was not clear. However, using a different RNA probe detecting a different region of HDV RNA, the 1.7-kb RNA species was clearly not detectable, even after prolonged exposure (Fig. 10C). (This RNA probe does not detect the 0.8-kb mRNA.) We therefore conclude that the 0.8-kb mRNA can be transcribed in the absence of HDV RNA replication.

DISCUSSION

In this paper, we have provided evidence that transcription of the HDV 0.8-kb mRNA and replication of the 1.7-kb antigenomic RNA are differentially regulated and require different cellular polymerases or the products of their transcripts. Synthesis of the 0.8-kb mRNA was sensitive to inhibition by α -amanitin at 3 μ g/ml, whereas synthesis of the 1.7-kb antigenomic HDV RNA was resistant to α -amanitin at concentrations as high as 25 μ g/ml. Furthermore, HDV mRNA synthesis can occur in the absence of 1.7-kb antigenomic RNA synthesis, and vice-versa, indicating that HDV RNA replication and mRNA transcription are not coupled. Thus, the mechanism of transcription of 0.8-kb mRNA is more similar to that of cellular pol II genes, whereas the synthesis of 1.7-kb RNA is significantly different. However, transcription of the 0.8-kb mRNA from RNA templates shows metabolic requirements distinct from those of DNA-templated transcription in several aspects; for example, an α -amanitin-resistant pol II mutant fails to completely restore the synthesis of 0.8-kb RNA and synthesis of the 0.8-kb HDV mRNA is suppressed at 34°C relative to the transcription of cellular mRNAs. Our results provided the first *in vivo* evidence for the sensitivity of HDV RNA-templated transcription to a low concentration of α -amanitin. This observed sensitivity clearly was not the result of the general cytotoxicity of α -amanitin, since the synthesis of the 1.7-kb HDV RNA species and cellular pol I- and pol III-mediated transcription were not inhibited under the same conditions.

The above findings lead to several conclusions about the regulation of HDV RNA replication and transcription. First, the cellular machineries that synthesize these two RNA species are different. All of the evidence supports the conclusion that the synthesis of the 0.8-kb mRNA species involves pol II or protein products of pol II-mediated transcripts. In contrast, the resistance of the 1.7-kb antigenomic RNA to α -amanitin at 25 μ g/ml suggests that it is synthesized by a polymerase other than pol II. The observed requirements for the synthesis of the 0.8-kb mRNA are consistent with the previous results of nuclear run-on experiments and *in vitro* reconstitution experiments (14, 31), although the *in vitro* experiments did not directly examine individual RNA species and their α -amanitin sensitivities. Furthermore, those experiments more likely reflect cDNA-templated transcription than true RNA-templated transcription (14, 31). In addition, the *in vitro* experiments did not require S-HDAg, which is a necessary factor in HDV RNA synthesis *in vivo*. Our experiments did not distinguish between the possibilities that pol II is directly involved in HDV RNA-templated transcription and that protein products of pol II transcripts participate in RNA transcription. Nevertheless, our

experiments provided unequivocal evidence that pol II is involved directly or indirectly in HDV mRNA synthesis but not in 1.7-kb antigenomic RNA synthesis. The finding that an α -amanitin-resistant mutant pol II failed to completely restore transcription of the HDV mRNA to wild-type levels (Fig. 8) suggests that there are clear differences between transcriptions from DNA and RNA templates. The α -amanitin-resistant mutant pol II is capable of transcribing most cellular mRNAs, as cells that express this mutant pol II had similar growth kinetics in the presence and absence of α -amanitin (after an initial lag). However, another α -amanitin-resistant mutant pol II (11) has been shown to cause selective reduction of transcription of certain cellular genes. Thus, the α -amanitin-resistant mutant pol II used in this study may also have selective defects for transcription from certain genes, including RNA-templated transcription.

The temperature dependence of 0.8-kb mRNA transcription also suggests that RNA-templated transcription has special requirements. One possibility is that temperature affects the conformation of RNA templates; it is possible that more extensive intramolecular base pairing of HDV RNA at the lower temperature prevented adequate unwinding of the template for transcription. Alternatively, temperature may affect the function of the genomic and/or antigenomic ribozymes, which is required for HDV RNA synthesis (24), or may interfere with the formation of an active transcription complex specific for RNA-templated transcription. In any case, the differential temperature sensitivity of DNA- and RNA-templated transcription further suggests that these two types of transcription processes have different requirements.

The failure of α -amanitin to inhibit the 1.7-kb antigenomic RNA synthesis was unexpected and suggests that cellular pol II is not involved in the RNA-templated synthesis of this RNA. Even exposure to α -amanitin for longer than 24 h did not inhibit its synthesis. This result contradicts the previous *in vitro* transcription data showing the sensitivity of 1.7-kb RNA synthesis to α -amanitin (14, 31). However, the *in vitro* studies were complicated by the presence of HDV cDNA, whereas our *in vivo* studies allowed us to focus exclusively on RNA-templated transcription. This conclusion raises an intriguing question regarding the identity of cellular enzymes responsible for HDV antigenomic RNA synthesis. pol I, pol III, or an as yet unidentified cellular enzyme may be responsible. The finding that the synthesis of the 1.7-kb RNA was sensitive to α -amanitin early after HDV RNA transfection but became resistant later suggests that the synthesis of this RNA is dependent on the availability of a large amount of HDAg. This interpretation was supported by the finding that the 1.7-kb RNA synthesis was not inhibited by α -amanitin at any time point after transfection in a cell line (Ts83) that constitutively expresses S-HDAg (Fig. 5B). The previous immunolocalization studies have shown that HDAg is not tightly associated with the pol II transcription machinery in the nucleus (12). Some HDAgs have been found to be localized in the nucleolus (3, 6, 43). Also, HDAg has been shown to interact with nucleolin (28), a nucleolar protein. These findings raise a possibility that pol I is responsible for HDV antigenomic RNA synthesis. This possibility is consistent with the resistance of the 1.7-kb RNA to high concentrations of α -amanitin.

The findings that 0.8-kb mRNA transcription can occur in the absence of 1.7-kb antigenome synthesis and vice versa further underscore the conclusion that HDV RNA transcription and replication have different metabolic requirements and are independent of each other. We showed that the 0.8-kb mRNA and HDAg were produced by a mutant HDV RNA which cannot synthesize the 1.7-kb antigenomic RNA. These

results suggest that replication of the genome requires a larger quantity of HDag than does the transcription of the 0.8-kb mRNA. Indeed, we found that when this mutant genome was transfected into Ts83 cells, which stably express S-HDag, HDV RNA replication occurred (L. E. Modahl and M. M. C. Lai, unpublished data). The requirement of newly synthesized S-HDag for genome replication is also consistent with the finding that the inhibition of 1.7-kb antigenome synthesis by α -amanitin early after transfection is secondary to inhibition of 0.8-kb mRNA synthesis (Fig. 3). However, the question remains as to the mechanism behind the differing requirements of S-HDag for mRNA synthesis and antigenome synthesis. S-HDag may be necessary to recruit transcription factors involved in the synthesis of the 1.7-kb antigenome. Indeed, both S- and L-HDag have been shown to inhibit transcription from a pol II reporter gene (30), suggesting that these proteins sequester factors required for pol II transcription. Alternatively, S-HDag may be necessary to maintain an HDV RNA structure specific for 1.7-kb antigenome synthesis. HDag is an RNA-binding protein which enhances ribozyme activity (25) and possesses an RNA chaperone activity (21). These properties may be required for successful replication of the HDV genome. The newly synthesized HDag may differ from the HDag provided either by transfected in vitro-transcribed HDag mRNA or HDag present in the HDV virion, thus explaining why newly synthesized HDag is required for 1.7-kb RNA synthesis.

In conclusion, the results presented here suggest that HDV RNA synthesis is different from plant viroid RNA replication in that synthesis of the different HDV RNA species directly or indirectly involves cellular pol II and other as yet unidentified cellular polymerases. Furthermore, HDag and other cellular factors are also required. The capacity of the mammalian cellular polymerases to carry out RNA-dependent RNA synthesis suggests an exciting new role for the cellular transcription machineries. Identification of the components of the machineries for RNA-templated RNA synthesis will be important for further understanding of these processes.

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